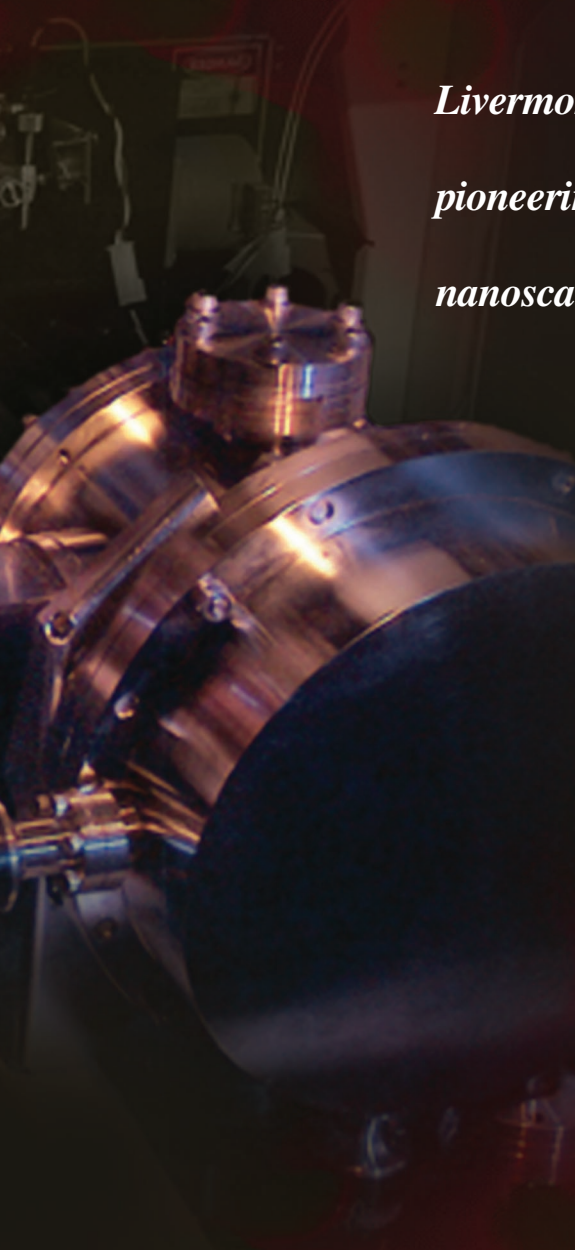
Life at

Sharon Shields, a chemist at Livermore's BioSecurity and Nanosciences Laboratory, uses a time-of-flight mass spectrometer to identify the proteins found in blood serum. These proteins are produced in response to the presence of pathogens.

the Nanoscale

Livermore's BioSecurity and Nanosciences Laboratory is pioneering new ways to detect pathogens and biomolecules at the nanoscale level and study how they function.



UNTIL recently, national security was synonymous with guns, tanks, and planes. Increasingly, however, the notion of security, especially homeland security, demands a broader meaning that includes ways to quickly detect and identify biological pathogens that might be unleashed by terrorists. Such pathogens could threaten urban population centers, crops, and livestock.

Livermore's BioSecurity and Nanosciences Laboratory (BSNL) is proving itself a national asset in the fight against bioterrorism by discovering new methods to detect, identify, image, and understand pathogens such as viruses, bacteria, and their spores. The research findings are also helping improve human health by providing a better understanding of pathogens and molecular machines such as DNA and proteins. In addition, BSNL researchers are contributing to the Department of Energy's Genomics:GTL Program, the follow-on effort to the Human Genome Project. The goal of Genomics:GTL (formerly called the Genomes to Life Program) is to understand the function of proteins and how they form the machines that drive the cells. Such information will help scientists better understand the complex biochemical activity of microbes.

BSNL's 62 researchers are drawn principally from Livermore's Chemistry and Materials Science Directorate, with significant contributions from the Biology and Biotechnology Research Program (BBRP); Engineering; Energy and Environment; Nonproliferation, Arms Control, and International Security; and Physics and Advanced Technologies directorates. More than half of the researchers are under 35 years old. "From the start, we adopted a strategy of investing in young talent, both from around the Laboratory and from scientists around the nation who are just starting their careers," says BSNL Director Jim De Yoreo.

The center has attracted four Lawrence fellows, who are some of the most sought after young Ph.D.s in the world. In addition, 27 students have worked with

BSNL scientists over the last three years; 11 student employees are currently doing their thesis work at BSNL. De Yoreo says the many young people create an environment where scientists do not hesitate to try new approaches and seek breakthroughs at the risk of failure.

Multidisciplinary research teams work at what De Yoreo terms the intersection of biology, chemistry, and materials science. Principal research areas are protein analysis and systems biology, bioaerosol science, molecular recognition chemistry, physical and chemical pathogen signatures (detection techniques), nanofabrication of devices, and cellular- and molecular-scale measurements.

A Natural Synergy

Founded in 1999 as the BioSecurity Support Laboratory, BSNL was reorganized in 2003 to increase its focus on what De Yoreo calls the "natural synergy" between nanotechnology and new frontiers in biological research. BSNL researchers work to exploit this synergy in three areas: sensing viruses, bacteria, and toxins; fabricating materials from the bottom up; and understanding the assembly and performance of protein machines and cellular systems.

Nanoscience takes its name from the nanometer, which is a billionth of a meter. BSNL researchers work on the nanoscale, or single molecule scale, to understand the organization of molecular complexes that make up most spores, viruses, DNA, or proteins—a level that provides unprecedented detail. (See *S&TR*, December 2001, pp. 12–19.)

At the nanoscale, experimental results can be viewed only with the most powerful imaging techniques, such as atomic force microscopy (AFM), confocal optical microscopy, and nano secondary-ion mass spectrometry. The two microsopy techniques can even image and manipulate single molecules, allowing researchers to study a molecule's structure and function. BSNL researchers' emphasis on single molecules differs greatly from that of traditional biological researchers, who examine beakers full of material and infer the actions of individual molecules.

Although electron microscopes have greater resolution, specimens must be frozen and covered with a metal film prior to imaging. With the optical methods used at BSNL, researchers can probe live cells to gain a much more realistic picture of their functioning and architecture.

Supporting some of the research is computer simulation. For example, BSNL scientist Andrew Quong used the Laboratory's ALE3D code to develop three-dimensional (3D) models that examine how epithelial cells communicate with each other. The simulations show strong agreement with experiments. (See *S&TR*, January/February 2003, pp. 15–18.)

The researchers are experts at synthesizing nanostructured materials such as artificial membranes with nanometer-size pores, microfluidic channels that guide the flow of single molecules for analysis, surfaces with nanometer-scale chemical patterns, and chemical compounds that recognize—or bind to—specific targets such as toxin molecules. Synthesis methods include using cells as chemical factories as well as traditional small-molecule techniques that can produce synthetic high-affinity ligands, which bind to pathogens and render them harmless. (See *S&TR*, June 2002, pp. 4–11.)

The First Signs of Disease

One of the most powerful tools used by BSNL scientists is the mass spectrometer, a device that measures the mass of individual molecules to precisely identify them. The instrument is being used to understand how cells respond when exposed to pathogens. The research is part of a pathomics project funded by Livermore's Laboratory Directed Research and Development Program. The term pathomics was coined by co-principal investigator Ken Turteltaub, a molecular biologist in BBRP. It is the science of applying proteomics, the study of proteins, to the discovery of certain proteins whose appearance or increase in concentration indicates a particular pathogen is present.

BSNL chemist Henry Benner says the research team ultimately wants to understand how individuals respond to pathogens, particularly those that bioterrorists might use. Such an improved understanding could lead to detection systems that identify an attack or disease outbreak before the first symptoms appear. "Our goal is to develop a technique that would analyze the proteins in a sample of blood serum and quickly detect the presence of a pathogen long before someone felt ill."

The project involves biologists, physicists, chemists, engineers, and computer scientists and uses some of the most sensitive mass spectrometers in the world. Says Benner, "This type of big, integrated project would be difficult to duplicate anywhere else."

The researchers are focusing first on human and animal serum proteins that are produced in response to vaccinia virus, the surrogate for smallpox virus. They are also collaborating with other institutions to determine how rodents can be used as model systems for human biochemical changes. In addition, they are studying how cell cultures can model the progression of a pathogen-caused disease.

Benner and chemists Sharon Shields and Chris Bailey have developed a liquid chromatography and mass spectrometry analysis system for characterizing plasma proteins in serum. Because most intact proteins are too large for the mass spectrometer to analyze, the team first applies an enzyme to the serum, which cuts each protein into about 50 chunks per molecule. The liquid chromatograph then separates and ionizes the protein chunks before they enter the mass spectrometer.

Detecting 10,000 Proteins

Benner notes that human blood serum can contain up to 10,000 different proteins, many of which are unknown. "With mass spectrometry, we analyze everything that's in the serum. We really don't need to identify every protein, although we'd like to eventually have that information. All we're looking for is a complex pattern—a series of mass spectrometry peaks—that corresponds to someone who is in the earliest stages of bacterial or viral attack and another pattern that corresponds to a healthy individual."

Almost certainly, different pathogens will produce different patterns. In this way, the researchers hope to accumulate a library of mass spectrometry patterns that identify specific pathogens. In addition, they must determine the extent to which various serum proteins vary in both normal and diseased individuals.

Another goal for the research team is to discover one or a few proteins that are a dependable signature for a pathogen's presence. This type of pathogen signature, based on proteins, differs from more traditional DNA-based signatures developed by Livermore researchers. (See *S&TR,* April 2004, pp. 4–10.)

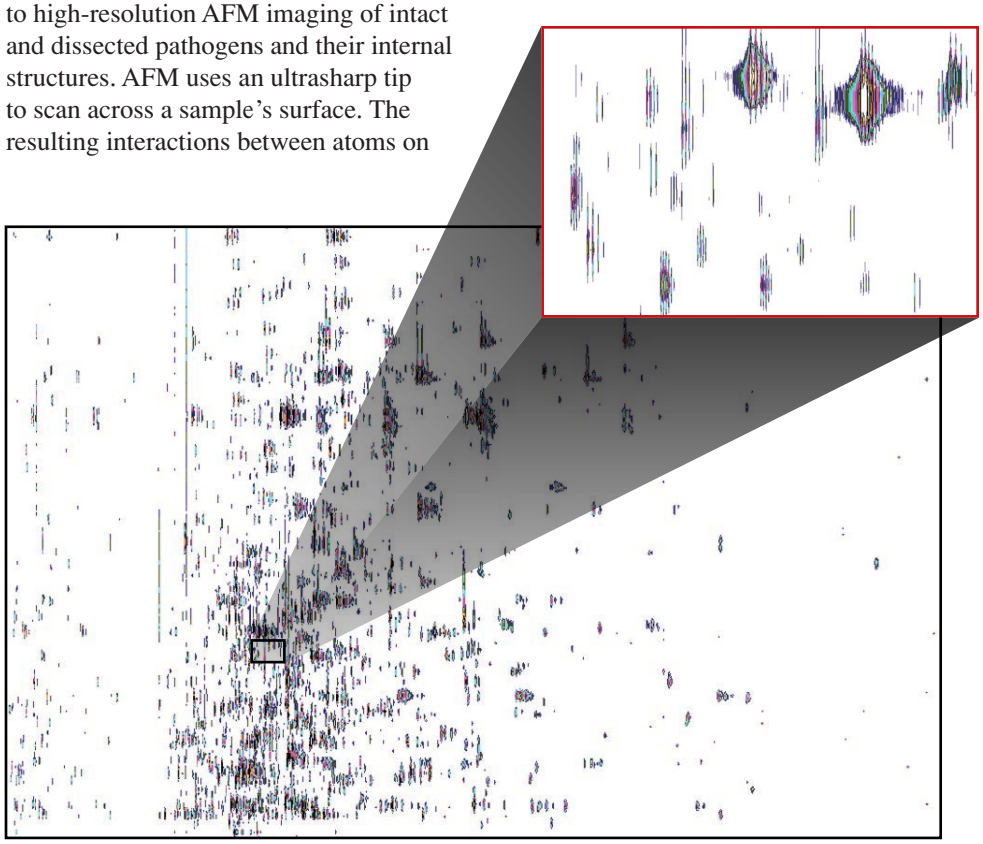
When perfected, the BSNL mass spectrometry technique would be valuable for detecting a bioterrorist attack, a natural outbreak of infectious disease, and even types of cancer. Another potential application is continual monitoring of the nation's blood supply. Patients receiving radiation treatment could also benefit from the technology because different mass spectrometry patterns would uniquely indicate damage to certain organs. A related project, led by scientists in BBRP and Livermore's Glenn T. Seaborg Institute, is using biomass spectrometry of blood serum to determine whether someone has been exposed to a dirty bomb—a crude nuclear device designed to cause widespread dispersal of radioactive materials.

Seeing Pathogens at the Nanoscale

Identifying and characterizing proteins that reside on the surface of human pathogens and that form their internal structures is critical to understanding how pathogens cause disease. Such information is also essential for developing vaccines and detectors for both medicine and biodefense. However, despite decades of study, scientists still have a poor understanding of many pathogens' structural properties. Common tools such as x-ray crystallography and electron microscopy often cannot be used because of some pathogens' large size, heterogeneity, and lack of symmetry.

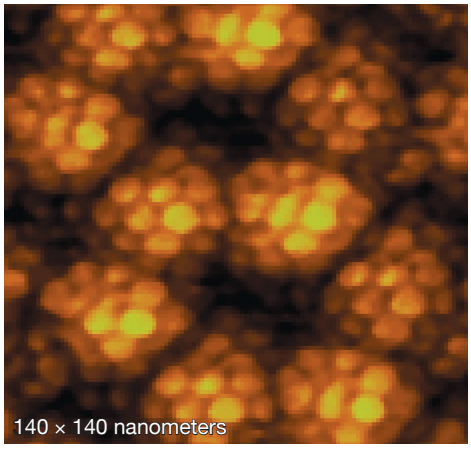
As a result, BSNL scientists have turned to high-resolution AFM imaging of intact and dissected pathogens and their internal structures. AFM uses an ultrasharp tip to scan across a sample's surface. The resulting interactions between atoms on the surface of the sample and those on the AFM tip are used to construct a high-resolution image of the surface topography.

BSNL chemists Alexander Malkin, Marco Plomp, and others are using AFM to image the proteins of intact human viruses and bacterial spores. They are focusing on bioterrorist threat surrogates such as the vaccinia virus, a laboratory model for smallpox virus. Vaccinia virus is one of the largest and most complex human viruses. The researchers are also studying several species and strains of innocuous *Bacillus* spores to understand the spore structure and function of *B. anthracis,* the agent of inhalation anthrax.



BSNL researchers use liquid chromatography to separate and ionize chunks of protein in a sample of blood serum and then analyze them with a mass spectrometer. The researchers hope to detect a pattern of mass spectrometry peaks (inset) that correspond to someone in the earliest stages of a particular bacterial or viral attack and another pattern that corresponds to a healthy individual. Each group of spots represents a single protein fragment.

For more than eight years, the researchers studied the molecular-scale mechanisms of crystallization for several types of proteins, viruses, nucleic acids, and ribosomes. Then using AFM, they imaged the high-resolution structure of these large ensembles of macromolecules. In work on agricultural viruses, Malkin imaged for the first time the structure of a small virus's capsid—the protein shell covering the viral genome—under physiological conditions. Image resolution approached an unprecedented 2 nanometers and clearly revealed the individual protein capsomeres that make up the capsid. (See the top figure below.) Malkin has demonstrated that viruses from different but closely related virus families can be differentiated by AFM on the basis of their capsid structure.



Atomic force microscope image of a crystalline array of turnip yellow mosaic viruses reveals the capsid structure, which can be resolved at high resolution.

Viral and Spore Structures

The scientists also imaged the Herpes Simplex Virus-1, one of the most widespread human viruses. This work demonstrated for the first time that the internal topography of viruses could be revealed by AFM using chemicals and enzymes to degrade particles from the outside to the inside, revealing each layer of the virus. Images showed the intact virus, the underlying capsid and its capsomere components, and finally, extrusion of viral DNA.

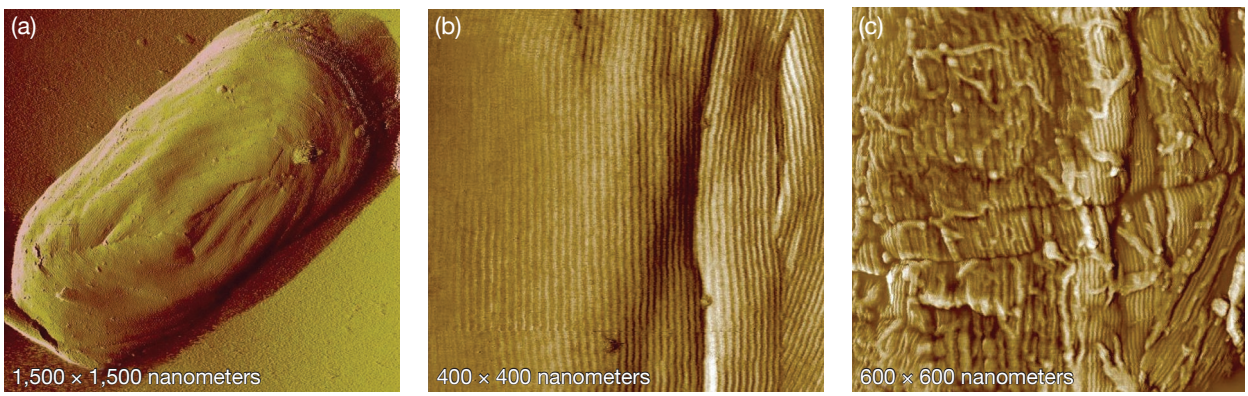
Evaluating the response of pathogens to the environment is important for understanding pathogen lifecycles and could help scientists develop detection systems and decontamination procedures. The researchers have visualized both hydrated and dehydrated samples of vaccinia virus. AFM images show that the surface of the hydrated form bristles with about 30-nanometer protein protrusions that had never been previously described for pox viruses. A membrane surrounds the viral core, which consists of 16-nanometer-diameter filaments containing double-stranded DNA. AFM visualization of intact viruses and their internal structures allows researchers to model the complex internal architecture of a large human virus.

Malkin and Plomp recently began visualizing bacterial spores. They resolved the surface structure of a *B. atrophaeus* spore coat and found that, in hydrated form, the top surface layer consists of regular arrays of rodlike crystalline structures that fold when dehydrated. (See the figure below.) In a study of two other *Bacillus* species, they found striking differences in spore structure. One species (*B. thuringiensis*) had an outer spore coat formed by a hexagonal honeycomb crystalline structure, whereas the other (*B. cereus*) had an outer rodlike structure and an underlying honeycomb structure.

The researchers are also pioneering a new approach called AFM-based immunolabeling. In this work, they use monoclonal antibodies synthesized to bind to targeted viral and spore proteins. The research is conducted in collaboration with scientists from the Oakland Children's Hospital Research Institute and the National Institute of Allergy and Infectious Diseases.

The mapping of surface proteins using monoclonal antibodies is a powerful tool for examining the surface topology of pathogens. Each bound monoclonal antibody defines one specific site on the antigen's surface. In this way, AFM can determine the location of proteins on a pathogen's surface—information that will

These images resolve the shape and surface features of a *Bacillus atrophaeus* spore. (a) A hydrated spore is magnified in (b), showing a surface consisting of arrays of rodlike structures that fold when dehydrated (c).



help scientists develop vaccines, detection systems, bioforensic methods, and decontamination procedures.

New Kinds of Sensors

One of BSNL's most important research goals is developing fast, sensitive, and accurate instruments to detect and identify a wide range of pathogens. In the area of airborne pathogen detection, Livermore researchers have worked with colleagues at the University of California (UC) at Davis to develop the bioaerosol mass spectrometer (BAMS). BAMS combines advanced laser desorption and ionization techniques with mass spectrometry, and it is two to three times more sensitive than other laser ionization techniques. In addition, the response time for BAMS is fast—it can identify a single airborne particle in about 100 milliseconds. (See *S&TR*, September 2003, pp. 21–23.)

Other researchers are working to shrink pathogen sensors to the size of a semiconductor chip for bioterrorism and health-care applications. BSNL physical chemist Alex Noy and graduate student Alex Artyukhin are developing a new kind of biosensor that is based on a lipid-coated nanotube, the first ever manufactured. In effect, the sensor is a tiny but mechanically resilient "molecular wire" designed to detect pore-forming bacterial toxins. These toxins, which are large proteins, are secreted by the bacteria and insert themselves into outer membranes of host cells. The 2-nanometer-wide holes created by the proteins rupture the cell and kill it.

To construct the biosensor, the researchers start with a carbon nanotube—a rolled-up, single layer of graphite. If the tube is rolled in a specific orientation, it becomes a semiconductor, a material that allows electrons to flow under certain conditions. Because of their electrical properties, semiconductors make excellent sensors.

With the help of microfabrication expert Olgica Bakajin, the researchers coat the nanotubes with a 5-nanometer-thick, dual-layer membrane made of phospholipids. The result is an insulated wire that mimics a cell membrane.

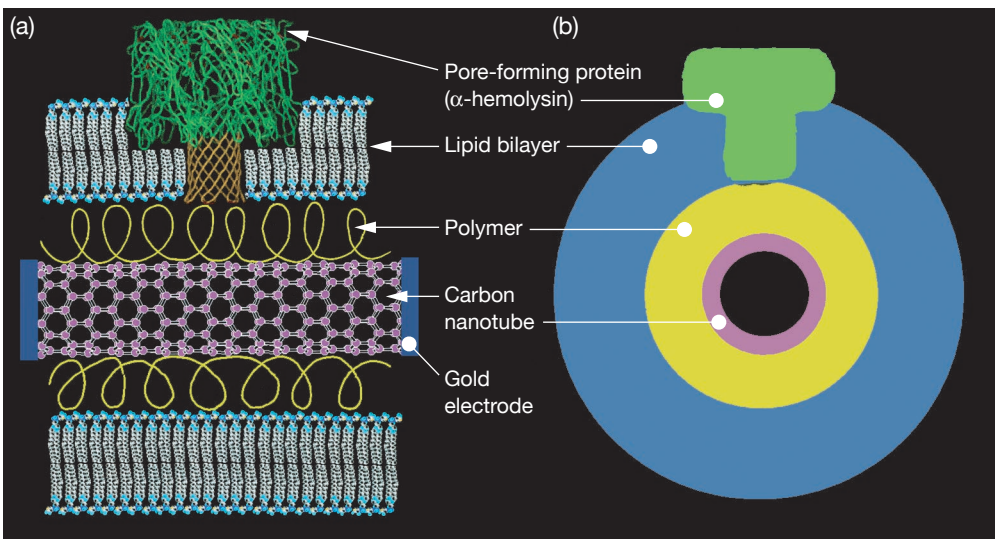
"Our idea was to construct something like a shielded cable that would be a good electrical detector," says Artyukhin. With electrodes attached to both ends of the nanotube and a voltage applied, the minuscule sensor can detect pathogen toxins that typically puncture a hole in a cell membrane or an artificial one. Any pathogen toxins in the immediate environment would insert themselves into the membrane. The punctures they create would allow ions to rush in, touch the bare nanotube, and immediately change the voltage.

The researchers recently added a polymer layer between the carbon nanotube and the lipid bilayer, to confer electrical stability and increase the tube's diameter. Many proteins require more room to function when they protrude through a membrane. The polymer layer can be reapplied to make several layers, each 1 nanometer thick. The researchers are experimenting with a five-layer nanotube.

Once perfected, the biosensor would be cheap to manufacture because it could be fabricated by the thousands, much like semiconductor chips in clean rooms. "Our biosensor is extremely simple," says Artyukhin. "It doesn't need lasers or other sophisticated equipment to function." The device would be ubiquitous and function as a "biological smoke detector."

A Close Look at Packaged DNA

AFM is central to a research effort headed by Noy that examines how DNA is packaged



A new type of biosensor is based on a lipid-coated nanotube: (a) longitudinal and (b) transverse sections. The sensor is designed to detect bacterial toxins such as the protein α-hemolysin, which pokes 2-nanometer-wide holes in cell membranes. The biosensor starts with a rolled-up carbon nanotube that is coated with a layer of polymer molecules and then a bilayer of phospholipids that mimic a cell membrane. With electrodes attached to both ends of the nanotube and a voltage applied, the minuscule sensor can detect pathogen toxins that puncture a hole in the membrane.

inside the cell nucleus so that it is a small fraction of its uncoiled size. He cites famed baseball player Yogi Berra, who once said, "You can observe a lot just by watching."

"Proteins mediate DNA packaging in all organisms," says Noy. Packaging protects DNA from physical damage and from free radicals, which are extremely reactive molecules. Different species package DNA differently. For example, mammalian sperm DNA is wrapped into dense toroids, like a pile of rope. But the mechanisms of how different proteins package DNA into distinct shapes are poorly understood.

Noy is focusing on the role of AbF2, a protein in yeast mitochondria—the cellular organelles that produce energy. AbF2's role is to "scrunch" DNA into a much more compact size. In collaboration with graduate student Ray Friddle and researchers at UC Davis, Noy has acquired AFM images of the DNA–AbF2 molecule. The images (below) show how the AbF2 binds to DNA and reveals the DNA–protein complex making repeated bends of 102 degrees.

"We then wondered whether the bends were important to compaction," Noy says. To find the answer, he constructed a computer model of the molecule, including the 102-degree bends. The model fit perfectly with data obtained from the images and showed that the bending indeed causes compaction. "When we put certain bends into DNA, it naturally folds into a compact shape," he explains.

An important lesson from this research is that studying and imaging a single molecule yields significant rewards. "When we use the single molecule technique," says Noy, "we get both binding information and the binding mechanism—the 102-degree bends. Traditional methods give binding information and then force us to deduce the mechanism."

Every new piece of data about a protein's structure and function helps efforts to detect, identify, and treat disease, says Noy. "We need to know what bad microbes do. The first rule is 'Know Your Enemy.'"

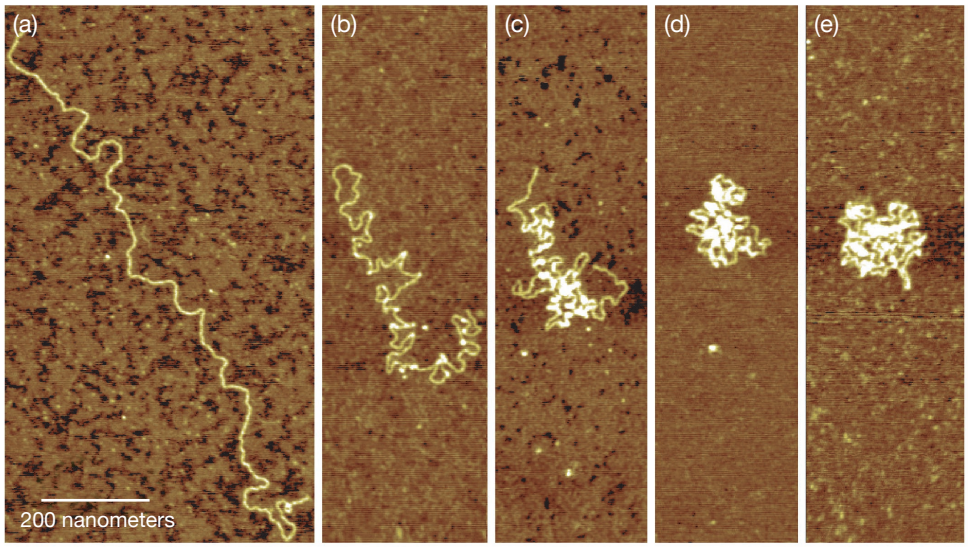
Probing Inside a Cell

BSNL physicist Thomas Huser and his colleagues Chris Hollars, Chad Talley, Anthony Esposito, and Stephen M. Lane have developed optical probes that enable nondestructive characterization and identification of cells and their functions at the nanometer scale. These probes use Raman scattering—the inelastic scattering of light by molecular bonds. Raman scattering is one of the few optical techniques that can identify a molecule by observing its distinct vibrational fingerprints as the molecule scatters laser light. (See the top figure on p. 11.) It also provides a more accurate representation than common fluorescent labeling of biomolecules. "With fluorescent techniques, we have to introduce fluorescent molecules that attach to the biomolecules of interest," says Huser. "Raman is the intrinsic signal from the native material."

He uses the confocal microscope, which is based on a fluorescence microscope augmented with a pinhole that limits the volume being probed and thus decreases background noise. The confocal microscope efficiently collects the scattered light emitted from molecules that have been excited by laser light. With this technique, Huser can perform Raman spectroscopy on single cells and look for differences between them.

The one drawback is that Raman spectra are quite weak. To increase the brightness and resolution of Raman-scattered light, Huser attached nanometer-size gold crystals to molecules or cells. The method, known as surface-enhanced Raman spectroscopy, increases the signal by a factor of a quadrillion (1×10^{15}) and vastly improves the sensitivity of the measurements.

Gold nanoparticles about 50 nanometers in diameter serve as tiny detectors that "report" on the environment they're in through Raman scattering. The particles are covered with molecules of mercaptobenzoic acid. Depending on pH, this molecule changes its Raman spectrum. "In essence, we've created an intracellular



(a–e) Progressive images from atomic force microscopy show the compaction of DNA in yeast caused by a protein called AbF2.

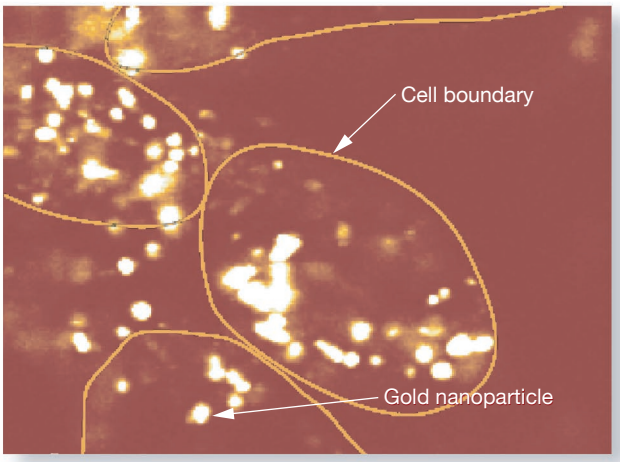
pH nanosensor that reacts to changes in its chemical environment," says Huser. If a cell undergoes changes as a result of external stimuli, its pH will usually change in response.

One possible application of this technique is studying the pH of cancer cells. Although tumors tend to be more acidic than normal tissue, the pH inside individual cancer cells is still mostly unknown. "We want to compare the pH of cancer cells to the exterior cell environment and to normal cells," Huser says. "We'd also like to see if cancer cell pH changes in response to different chemotherapy agents." Another possibility is to place the nanoparticles just outside the cells to signal the presence of certain proteins belonging to pathogens.

Huser and his colleagues are applying their expertise in a Genomics:GTL project, using nanoprobes to study how microbes clean up the environment by digesting toxic molecules. "Microbiologists would like to obtain much more detailed information about how some microbes assimilate toxic materials," he says.

Huser and Hollars are also part of a new effort, headed by Bailey, to study a class of mysterious proteins called prions. When misfolded, prions can attack healthy cells. Prions cause mad cow disease, technically known as bovine spongiform encephalopathy, and humans can contract a similar form, known as variant Creutzfeldt–Jacob disease. In sheep, prions cause a degenerative disease called scarpie.

In collaboration with the U.S. Department of Agriculture, the team is developing techniques to look for prions in sheep blood serum. One approach is to add fluorescent molecules that would bind to any prions. The serum is then run through microfluidic channels that are 100 micrometers wide, 500 micrometers long, and 0.5 micrometer deep. An optical microscope, sensitive to fluorescence, would detect any prions.



Scientists are using gold particles measuring 50 nanometers in diameter as cell pH sensors. The particles are coated with molecules of mercaptobenzoic acid, which changes its Raman spectrum in response to changes in the chemical environment.

BSNL chemist Chad Talley (left) and physicist Thomas Huser use a fluorescent microscope with a wide field of view to image single molecules.

Meeting the Vision

By any standard, says De Yoreo, BSNL is meeting its goals to become a formidable resource for advancing national biosecurity, improving human health, and understanding the molecular machinery of life. Increasing funding from sponsors, a growing number of publications in major peer-reviewed journals, and deepening scientific understanding of pathogens and biomolecules all speak to its success. The next few years should bring even greater understanding of life processes at the nanoscale.

—Arnie Heller

Key Words: atomic force microscopy (AFM), biodefense, BioSecurity and Nanosciences Laboratory (BSNL), bioterrorism, DNA, Genomics:GTL Program, nanoparticles, nanoscale, nanotube, pathogens, pathomics, prions, proteomics, signatures, smallpox.

For further information contact Jim De Yoreo (925) 423-4240 (deyoreo1@llnl.gov).